Astringency - A Molecular Model for Polyphenol/Protein binding

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Introduction

Astringency is recognized as a feeling of dryness, constriction and puckeriness of the palate, which takes a significant time to develop. It is caused by the intake of food, like grapes, dates or beverages, like tea, coffee, red wine or beer. All those beverages or food have in common that they contain plant polyphenols. Polyphenols are secondary metabolites, widely distributed in the plant kingdom and playing an important role in their defence mechanism [1]. It is commonly accepted that the astringent response is the result of polyphenols precipitating salivary proline-rich proteins (PRPs), which are part of the mucous surface layer of the mouth. The consequence is a loss of lubrication, which is generally perceived as an astringent sensation. In order to understand the mechanism of the astringent response, attention needs to be drawn to the complexation between plant polyphenols and salivary PRPs [2]. Since this is a complex process, a variety of techniques were applied to investigate different aspects of the binding process.

In this study β-casein was used as a model protein for PRPs and (-)-epigallocatechin gallate (EGCG) was chosen as a representative for polyphenols. Transmission electron microscopy and analytical ultracentrifugation demonstrate the polydispersity and the size distribution of the system, and analytical unltracentrifugation shows that as the proportion of EGCG is increased, the larger particles increase in both number and size. Small-angle X-ray scattering and dynamic laser light scattering show that there is an initial compaction of the protein as it coils around the polyphenol, and that aggregation sets in as the concentration of EGCG is increased, leading finally to precipitation. As a result of this investigation and supported by previous studies [3, 4] a binding model consisting of three steps for the interaction between salivary proline-rich proteins and polyphenols was developed.

Experimental Procedure

As PRPs are difficult to purify to homogeneity, β-casein, which is readily available, was chosen as a model protein due to its conformational similarities with PRPs. β-casein is a protein of the mammalian milk and possesses 35 prolines evenly distributed throughout the amino acid sequence. In its original state it is an amphiphilic molecule due to five phosphoserine groups concentrated on the N-terminal [5, 6]. However, by enzymatic dephosphorylation the tendency to form micelles is suppressed.

(-)-epigallocatechin gallate (EGCG) is a typical constituent of many foodstuffs and beverages and was therefore chosen as ligand for the polyphenol/protein interaction. It belongs to the group of condensed proanthocyanidins and possesses three aromatic rings, which act as potential binding sites [7].

The solvent used was H_2O:DMSO (95:5 by volume) and all experiments were conducted at a pH of 7.0 ± 0.2. The concentration of EGCG was generally 10 mmol l^{-1}. and it was mixed with β-casein at different ratios.

Transmission electron microscopy (TEM) - β-casein (4.2 x10^{-6} mol l^{-1}, 0.1 mg ml^{-1}) was mixed with EGCG in a molecular ratio of 1:1 and immediately after mixing fixed on a carbon grid and stained with uranyl formate. The sample grids were then examined using a Philips CM100 transmission electron microscope at an accelerating voltage of 100 kV.

Analytical Ultracentrifugation (AUC) - Measurements were conducted using an Optima XL-I analytical ultracentrifuge (Beckman Scientific Inc., Palo Alto, USA) at the National Centre for Macromolecular Hydrodynamics at the University of Nottingham with a β-casein concentration of 4.2 x 10^{-5} mol l^{-1} (1.0 mg ml^{-1}). The experiments were performed applying the sedimentation velocity method and a run speed of 50,000 rpm. The data were recorded as concentration versus radial position (r) and sedimentation coefficients were determined using the time derivative software (DCDT+) developed by Dr. J. Philo (Biotechnology & Software Consulting, Thousands Oaks, USA) [8, 9].

Small angle X-ray scattering (SAXS) - β-casein, at a concentration of 4.2 x 10^{-4} mol l^{-1} (10.0 mg ml^{-1}) was mixed at different ratios with EGCG. The measurements were carried out on beamline 2.1 at the SRS Daresbury laboratory. The sample time was limited to 60 s because the protein was subject to radiation damage. The samples were measured at two camera lengths: 2.25 and 8.0 m, and subsequently the data were merged in order to fit the scattering curves using the program GIFT [10].

Dynamic laser light scattering (DLS) - β-casein was filtered using a Minisart sterile filter with a pore size of 0.2 mm. The concentration was determined by UV spectrophotometry as 1.8 x 10^{-4} mol l^{-1} (4.2 mg ml^{-1}). The measurements were performed using a BI-200SM Goniometer version 2.0 from Brookhaven Instruments Corporation with Brookhaven Instruments Particle Sizing Software ver. 3.42 and measurements were conducted at an angle of 90°. A lognormal fitting procedure was applied to fit the time correlation function to a single particle size.

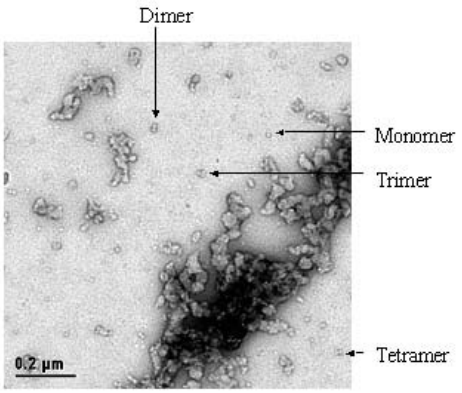

Figure 1. TEM micrographs of β-casein mixed with EGCG in the ratio 1:1 shows the polydisperse nature of the β-casein/EGCG mixtures. Monomers, dimer, trimers and higher aggregates can be seen.

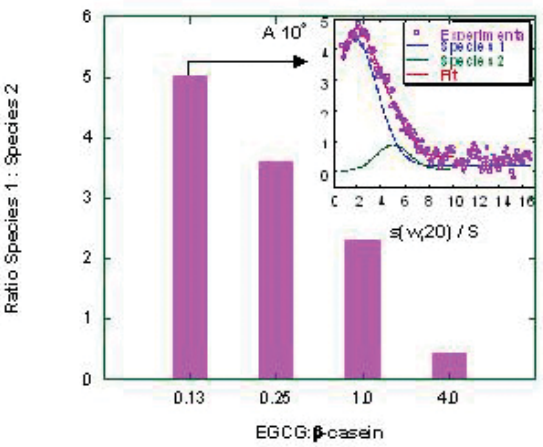

Figure 2. Analytical ultracentrifugation measurements show that the ratio of species 1 (s~2 S) to species 2 (s~5S) decreases significantly as the concentration of EGCG:β-casein is increased from 0.125:1 to 4:1. This is attributed to the progressive aggregation of protein molecules caused by the addition of EGCG. Inserted are the experimental data from the mixture EGCG:β-casein 0.125:1 and their best fit. The curve can be fitted to two species, one exhibiting a sedimentation coefficient around 1.85 S and the larger species around 5.1S. Furthermore a very large aggregated species is present which however, migrates very rapidly to the bottom of the centrifuge tube and therefore cannot be measured.

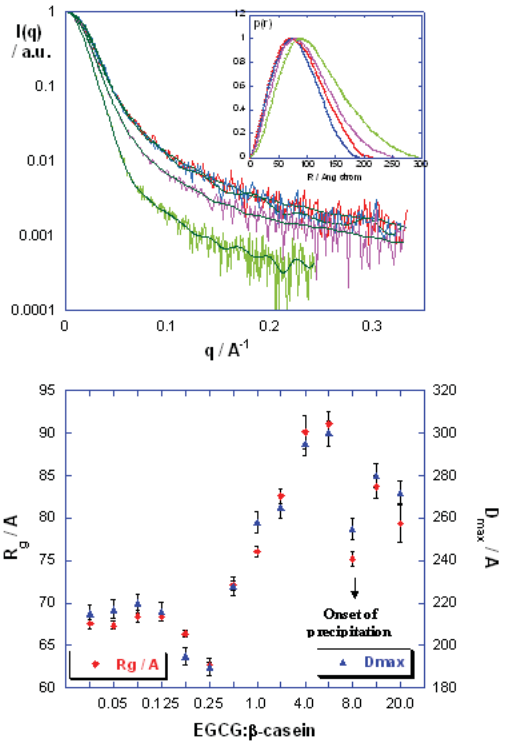

Figure 3. The normalized scattering curves and the corresponding pair distance distribution functions p(R) (inset) for β-casein mixed at different ratios with EGCG are shown. Red: β-casein, blue: EGCG:β-casein 0.25:1, purple: EGCG:β-casein 1:1, EGCG: β-casein 4:1. (Top). R_g and D_{max} exhibit a minimum value at a ratio EGCG:β-casein 0.25:1, due to increased coiling up of the protein around the polyphenols. As the EGCG concentration is increased, it starts to cross-link different proteins, which finally lead to precipitation. (Bottom)

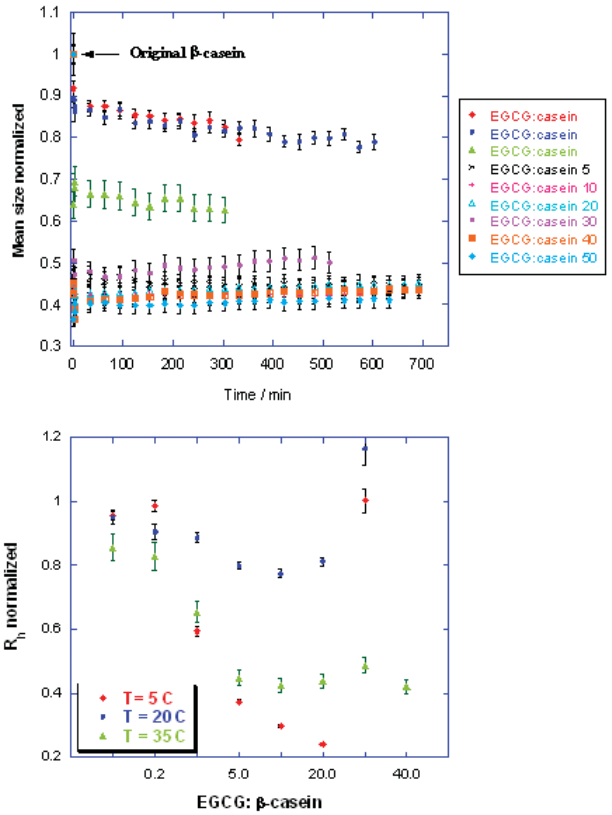

Figure 4. Dynamic laser light scattering (DLS) data show that the compaction of the β-casein molecule occurs within the first minute after mixing with EGCG, and thereafter there is little change in the particle size distribution. (Top) Dependence of particle size measured by DLS on EGCG:β-casein ratio, at three different temperatures. β-casein mixed at different ratios with EGCG shows initially a reduction in the hydrodynamic radius (R_h), which as the EGCG ratio is increased, rises due to complexation and aggregation. (Bottom)

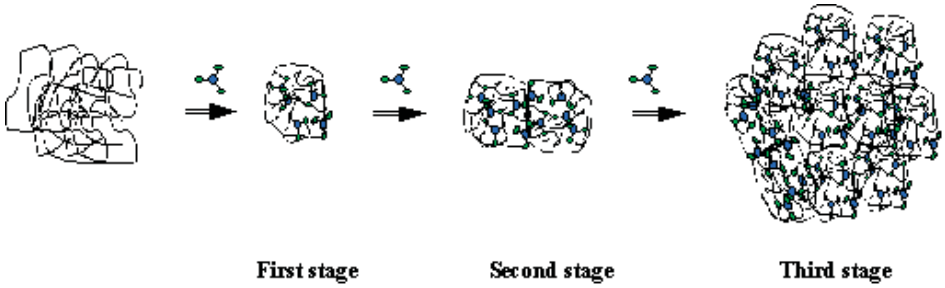

Figure 5. The proposed molecular binding model for the interaction of salivary proline-rich proteins with polyphenols. In the initial stage the proteins compact, attributed to tight and multiple binding to the muiltidentate polyphenols. At the second stage a dimer of polyphenol coated protein is formed, rendering the complex insoluble. At the third stage, further complexation leads to large complexes and precipitation of the protein.

Results

Transmission electron micrographs (Fig. 1) show the polydisperse nature of the β-casein - EGCG mixtures. Monomers, dimers, trimers and higher aggregates can be distinguished, whereby the monomers can be identified as spherically shaped.

In order to fit the analytical ultracentrifugation data adequately, it is necessary to include at least 2 species. It is of course likely that more than 2 species are present and it is assumed that each species in fact represents a distribution of particle sizes or shapes. The two species obtained from the fit have sedimentation coefficients of approximately 2 S and 5 S respectively. The ratio of species 1/species 2 when increasing the EGCG concentration shows a dramatic decrease indicating that at higher EGCG:β-casein ratios the number of the larger aggregates increases at the expense of monomers and smaller aggregates (Fig. 2).

Fig. 3A shows the normalized scattering curves and the corresponding pair distance distribution functions p(R), which yield the value of the maximum molecular dimension D_{max}. Fitting of the scattering curves gives a value for the radius of gyration R_g of the β-casein molecules and from Fig. 3B it can be seen that at an EGCG:β-casein ratio of 0.25:1, R_g as well as D_{max} decrease caused by the compaction of the protein molecules. The minimum in particle size is followed by an increase, due to aggregation. As soon as the complexes become insoluble and precipitation occurs the data start to become unreliable. The p(R)-function gives an indication of the particle shape. The compact β-casein at low EGCG concentrations gives rise to a symmetric p(R)-function, illustrating that the protein adopts a rather spherical conformation compared to the elongated original structure [11]. Similar conclusions are reached by analysis of Kratky plots (data not shown).

Astringency is a sensation that develops over the course of approximately 30 seconds, and then is gradually lost, due to continual salivary flowing washing away complexes of PRPs [12, 13]. Dynamic laser light scattering (DLS) was therefore applied to measure the hydrodynamic radius (R_h) of the proteins shortly after mixing with polyphenols. The DLS results (Fig. 4A) show that the compaction of the β-casein molecules occurs within the first minute after mixing and the hydrodynamic radius (R_h) then stays constant for hours. Fig 4 B shows that R_h passes through a minimum at a ratio EGCG:β-casein ~ 10:1. This ratio, where the protein adopts the minimum size, is significantly higher compared to that observed in the SAXS data (0.25:1). This may be on the one hand explained by the way the different techniques average over polydisperse distributions. On the other hand it is likely that much of the difference is due to the fact that there are no β-casein aggregates present at the start of the DLS experiments, since DLS measurements require careful filtering of the solutions prior to measurements in order to remove dust particles, which would influence the scattering data greatly. It is therefore suggested that the presence of casein aggregates induces particle aggregation at lower EGCG:β-casein ratios; in other words, EGCG-coated monomers appear to bind preferentially to casein aggregates.

Discussion

This study aims to develop a molecular model for the interactions between salivary basic proline-rich proteins and dietary polyphenols, which are most likely responsible for the astringent sensation caused by polyphenol-rich foods and beverages. As a conclusion from the experimental results a binding model in three steps is suggested (Fig. 5). The association of polyphenols with proteins is principally a surface phenomenon. Polyphenols are multidentate ligands, whereby each phenolic ring is a potential binding site [14]. Therefore they are able to bind at more than one point on the protein stand. Multiple non-covalent bonds, mainly hydrophobic interactions complemented by hydrogen bonding, are responsible for the interactions of polyphenols with proteins [15].

At the first stage and at low EGCG concentration the polyphenol associates onto the protein surface and generally several polyphenol molecules bind simultaneously and tightly to the same protein. This causes the protein, which originally exists in a loose randomly coiled conformation, to 'wrap' around the polyphenols hence experiencing a compaction of its structure. At the second stage EGCG starts to cross-link different proteins leading to the recruitment of a second polyphenol-coated protein, which renders the complex insoluble, forming a colloidal solution. At the third and final stage, further cross-linking causes the aggregates to grow, finally leading to precipitation [3, 16].

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